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Self-Cleaving Circular RNA Associated with Rice Yellow Mottle Virus Is the Smallest Viroid-like RNA

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We report the sequence, structural features, and self-cleaving activity of the small circular RNA (sc-RNA) associated with rice yellow mottle sobemovirus (RYMV). At 220 nucleotides, the RYMV sc-RNA represents the smallest naturally occurring viroid-like RNA currently documented in the literature. It is similar to other circular satellite RNAs (sat-RNAs) and viroids in being G-C-rich with a high level of self-complementarity. The predicted native structure is essentially a rod with one branched terminus. A region of the RYMV sc-RNA, constituting 24% of the sequence, exhibits 89% identity to the sat-RNA associated with the Australasian isolates of lucerne transient streak sobemovirus. This region is also structurally similar in all three RNAs in that it forms the left terminus of each rod. Dimeric runoff transcripts of cloned RYMV sc-RNA undergo efficient autocatalytic *in vitro* cleavage in the (+) but not the (−) polarity. Analysis of the (+) sequence indicates the presence of a hammerhead ribozyme resembling that of carnation small retroviroid-like RNA and the genomic satellite transcript of newt. Inefficient cleavage of (+) monomeric transcripts, and a short stem III in the hammerhead, are features consistent with a double-hammerhead mode of self-cleavage. The presence of sat-RNA and retroviroid-like structures within a single RNA suggests a possible role for the RYMV sc-RNA as an evolutionary intermediate between these subviral RNAs.

INTRODUCTION

Rice yellow mottle sobemovirus (RYMV) is widespread on cultivated rice in many countries of western and eastern Africa (Hull, 1988; Thottappilly, 1992), including the island of Madagascar (Reckhaus and Randrianangaly, 1990). RYMV virions are isometric with 28-nm particles (Bakker, 1975) and package a 4.5-kilobase single-stranded, messenger-sense viral RNA (Yassi *et al.,* 1994). The genomic organization of RYMV is similar to other sobemoviruses for which complete sequences are known (Yassi *et al.,* 1994). Virions of some isolates of RYMV also encapsidate a low-molecular-weight RNA which has satellite-like properties. Although this purified low-molecular-weight RNA was shown to be infectious when co-inoculated with RYMV genomic RNA but noninfectious alone, the satellite nature of this small RNA has not been unequivocally demonstrated, and thus it has been designated a small RYMV-associated RNA (Sehgal *et al.,* 1993).

In previous work, the small RYMV-associated RNA was observed by electrophoresis to be present in both circular and linear forms (Sehgal *et al.,* 1993). Its size was estimated to be 210 nucleotides (nt), and Northern hybridization indicated sequence identity to a larger (322-nt) circular satellite RNA (sat-RNA) associated with a Canadian isolate of lucerne transient streak sobemovirus (LTSV; AbouHaidar and Paliwal, 1988). This LTSV sat-RNA shares 80% sequence identity to the sat-RNAs associated with two Australasian isolates of LTSV (Keese *et al.,* 1983) but has little identity to the sat-RNAs of three other sobemoviruses (reviewed by Francki, 1987; Symons, 1991). Despite the general lack of sequence similarity among these circular satellites, all are structurally similar to viroids in that their native secondary structures are predicted to form mostly double-stranded rods with short terminal branches (Francki, 1987; Gast *et al.,* 1996). Additionally, all of the sobemovirus-associated sat-RNAs contain hammerhead ribozyme motifs believed to be involved in autocatalytic processing during a rolling circle mode of replication (Symons, 1991).

We now present the complete nucleotide sequence and *in vitro* cleavage of the small, circular RYMV-associated RNA (sc-RNA). In a recent survey of all reported viroid and viroid-like RNA sequences (Bussiere *et al.,* 1996), the sizes of such circular RNAs range from 246 to 463 nt. At 220 nucleotides, the RYMV sc-RNA represents the smallest naturally occurring viroid-like RNA currently described in the literature. Moreover, sequence and structural similarities of the RYMV sc-RNA to the viroid-like sat-RNA of LTSV, the carnation retroviroid-like RNA (CarSV; Daros and Flores, 1995), and the genomic satel-

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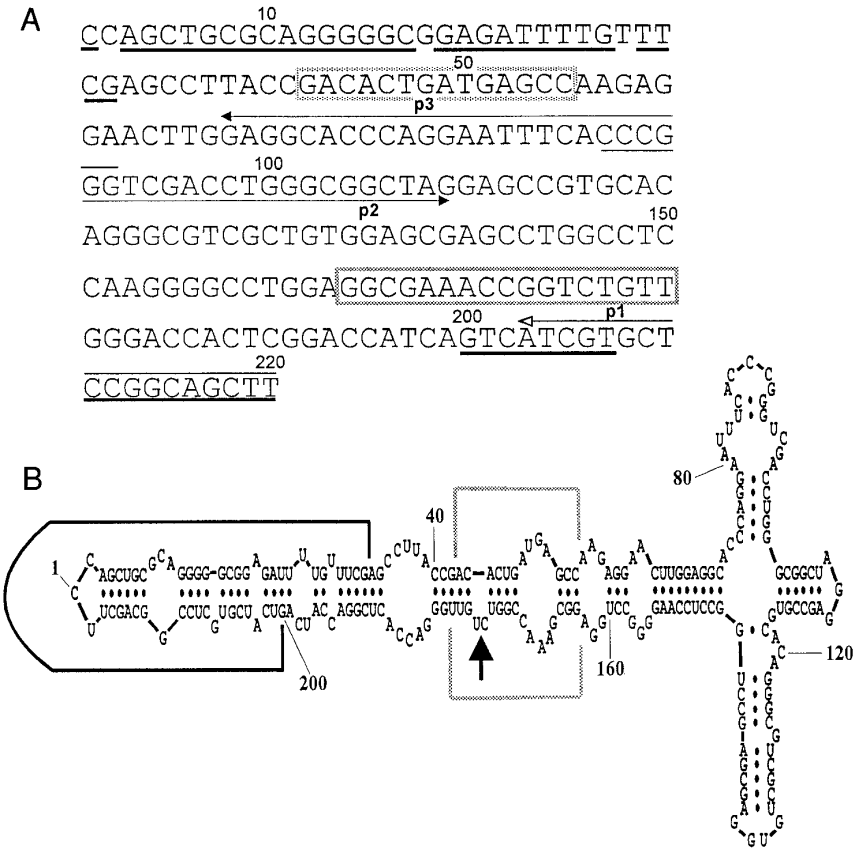

FIG. 1. Nucleotide sequence and predicted structure of the RYMV sc-RNA. (A) Complete 220-nt, (+) sequence. Polarity and numbering assigned according to the LTSV sat-RNA, based on the pattern of identity between these RNAs. (B) Predicted secondary structure of circular sequence. Thick solid line, region with 89% identity to the Australasian isolates of LTSV sat-RNA (nonidentical nucleotides within this region are not underlined); hatched boxes, sequences involved in forming (+) hammerhead; thin solid arrows, primer binding sites for primers (p2 and p3) used for RT-PCR; thin unfilled arrow, binding of primer (p1) used for primer extension to determine cleavage site; large arrow, the cleavage site.

lite transcript of newt (Epstein and Gall, 1987) suggest an evolutionary relationship with other RNA replicons.

RESULTS AND DISCUSSION

Sequence of the RYMV sc-RNA

The complete 220-nt sequence obtained (Fig. 1A) is derived from six separate clones (*i.e.,* one full-length clone generated by PCR and five partial-length clones) that were originally generated from two methods of producing cDNA. Random DNA hexamers were initially used to prime cDNA synthesis. This produced five partial overlapping clones which represent a circular sequence with no identity to genomic RYMV. Back-to-back (sense and antisense) primers based on this sequence were then used for RT-PCR, producing full-length monomeric cDNA from fractionated RNA. This monomeric cDNA was cloned into a vector (pBS(+)) allowing transcription of both strands, and one clone was sequenced and subsequently used to produce a head-to-tail dimer in the same transcription vector. No sequence heterogeneity was found among any of the clones.

The RYMV sc-RNA sequence is similar to other circular sat-RNAs and viroids in that it has a high G-C content (63.6%). The predicted lowest free-energy secondary structure ($\Delta G = -78.6$ Kcal/mol at 37°C), generated using the computer program MFold (Zucker, 1989), is essentially a rod with a branched right terminal structure (Fig. 1B). Sixty-seven percent of the nucleotide positions are internally base-paired; such a high level of self-complementarity is prevalent among the viroids and other circular sat-RNAs associated with sobemoviruses.

One region of the sequence, representing 24% of the RYMV sc-RNA, has 89% identity to the highly conserved sat-RNAs associated with the Australian and New Zealand isolates of LTSV (Fig. 1). Moreover, this region has a similar structural position in both RNAs: a 50-nt hairpin loop that represents the left terminus of the rod. Such a high level of domain-like identity is not shared between the LTSV sat-RNA and any other reported sat-RNA or viroid-like RNA. Additionally, 14 nucleotides located in a central region of the RYMV sc-RNA rod (Fig. 1B) are highly conserved among RNAs which have a hammerhead ribozyme (Fig. 4). There is otherwise no identity to sequences currently registered with GenBank (using the NCBI-BLAST search strategy).

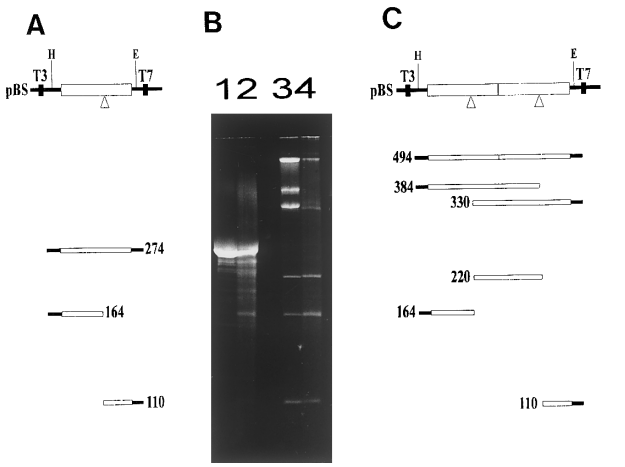

FIG. 2. *In vitro* cleavage of RYMV sc-RNA (+) runoff transcripts. (A and C) Schematic maps of monomeric and dimeric cDNA clones and expected cleavage products. (B) PAGE (in presence of 7 M urea) of monomeric (lanes 1–2) and dimeric (lanes 3–4) transcripts either before (lanes 1, 3) or after (lanes 2, 4) incubation under cleavage conditions. Arrows mark location of the cleavage site. Solid line indicates vector sequences. Promoters for T3 and T7 RNA polymerase are indicated as filled boxes. H, *Hin*dIII site; E, *Eco*RI site.

Analysis of the (−) polarity sequence found three small possible open reading frames (ORFs) distributed across the three reading frames. A similar search of the (+) sequence indicated one possible ORF. Intriguingly, the AUG of this unique (+) ORF overlaps two tandemly repeated UGA codons such that both stop codons occur two frameshifts from the initial reading frame (*i.e.,* with no stops in the intervening reading frame). Translation of this putative ORF would thus terminate only after two complete rounds of translation and would require a multimeric or circular RNA template to produce a 146-amino-acid polypeptide (currently under investigation).

The smallest previously reported viroid or viroid-like RNAs are the 246-nt isolates of avocado sunblotch (ASBV) and coconut cadang cadang viroids (CCCV; Bussiere *et al.,* 1996). The RYMV sc-RNA, at 220 nucleotides, is thus the smallest among the viroid-like RNAs reported to date. Presumably, a minimum number of nucleotides are required by a circular RNA to maintain the integrity of the *in vivo* native structure(s) that contributes to overall stability and biological activity. The 24% of the RYMV sc-RNA sequence that has identity with the LTSV sat-RNA may be important for replication, while another 20% appears to be involved in forming a hammerhead ribozyme (Fig. 4A). It will be of interest to see if even smaller circular RNAs naturally occur.

Cleavage of satellite transcripts

In vitro runoff transcription to produce (+) dimeric RNAs, followed by treatment with the conditions known to induce autocatalytic cleavage in hammerhead ribozymes, demonstrated that the RYMV sc-RNA (like other sobemovirus sat-RNAs) is capable of self-cleavage (Fig. 2B, lane 4). Moreover, this activity is very efficient in (+) dimer transcripts; nascent RNAs, treated only under transcription reaction conditions, also produced cleavage products (Fig. 2B, lane 3). By comparison, monomeric (+) transcripts cleaved with low efficiency, producing only a small amount of cleaved product upon treatment under cleavage conditions (compare lanes 2 and 4, Fig. 2B).

The position of the cleavage site, between nucleotides C_{176} and U_{177}, was determined by runoff primer extension of cleaved transcripts (Fig. 3). Analysis of the sequence around this site indicated a hammerhead ribozyme could be formed in the (+) polarity (Fig. 4A), with the required consensus nucleotides in their expected positions. The resultant hammerhead has a short stem III stabilized by only two base-paired nucleotides (Fig. 4A). Comparison with ribozymes of other self-cleaving RNAs indicates structural similarities to the hammerheads predicted for CarSV and the genomic satellite transcript of newt (Figs. 4B and 4C). The presence of a shortened stem III makes this structure unstable and suggests a 'double' hammerhead mode of cleavage may occur (Fig.4D); such a model has previously been proposed for both the CarSV and the newt ribozymes (Forster *et al.,* 1988; Hernandez *et al.,* 1992). Our observation that the (+) dimer of the RYMV sc-RNA is more efficiently cleaved than the (+) monomer is consistent with a double-hammerhead model of cleavage.

Incubation of either dimeric or monomeric (−) transcripts under cleavage conditions did not release a detectable level of cleavage products (data not shown).

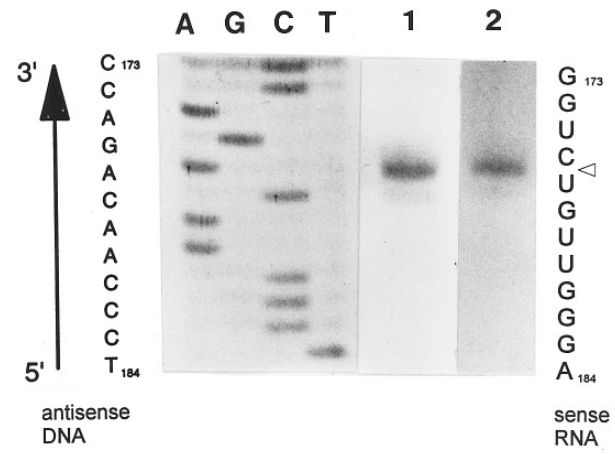

FIG. 3. Primer extension on cleaved RYMV sc-RNA transcripts. PAGE (in the presence of 7 M urea) of [α-^{35}S]dATP-labeled DNA fragments produced by runoff reverse transcription of cleaved transcripts. Lanes A, G, C, and T are sequencing reactions of dimer clone, using the same 18-mer primer (p1) for the extension reactions (see Materials and Methods). Arrow indicates direction of primer extension. Sequence is indicated with numbers corresponding to nucleotide position in sc-RNA. Lane 1, primer extension using eluted 164-nt 3′ end RNA fragment. Lane 2, primer extension using eluted 220-nt fragment (see Fig. 2C).

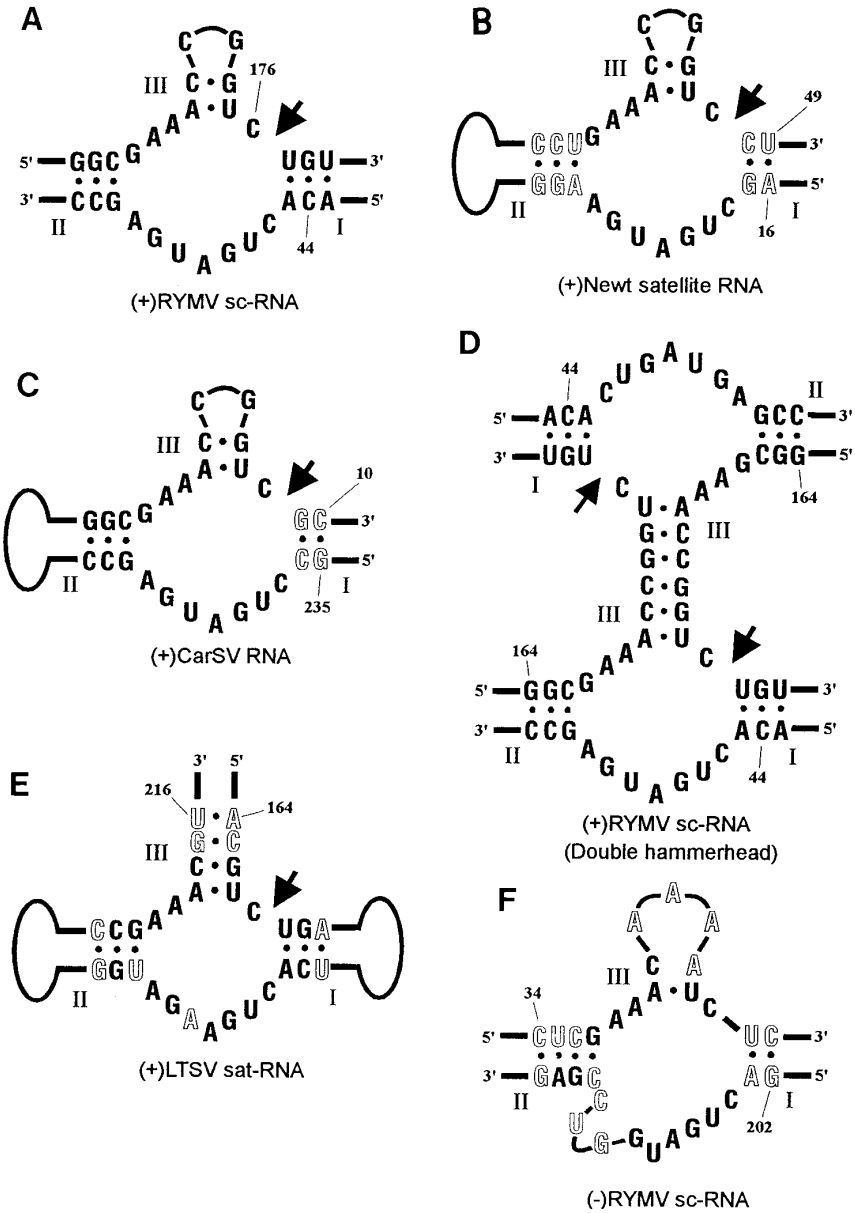

FIG. 4. Predicted structures of hammerhead ribozymes from various RNAs. (A) (+) RYMV sc-RNA. (B) Genomic satellite transcripts of newt (Epstein and Gall, 1987). (C) (+) CarSV RNA (Hernandez *et al.,* 1992). (D) Double hammerhead of (+) RYMV. (E) (+) LTSV sat-RNA (AbouHaidar and Paliwal, 1988). (F) (−) RYMV sc-RNA. Arrows mark cleavage sites. Filled bases indicate identity with RYMV sc-RNA. Nucleotide positions are according to the original numbering of the sequence reported. Roman numerals indicate stem numbering according to consensus hammerhead structure.

Analysis of the (−) sequence indicated that, despite the presence of nucleotides that are conserved in hammerhead ribozymes, a stable hammerhead structure could not be formed (Fig. 4F). It is conceivable that the presence of these conserved nucleotides, but lack of ribozyme activity and a hammerhead structure, may indicate a newly emerging hammerhead or a vestige of a previously functional hammerhead in the (−) polarity. Self-cleavage in the (+) but not the (−) polarity indicates that the RYMV sc-RNA likely replicates according to an asymmetric rolling circle pathway (Forster and Symons, 1987). This is similar to the sat-RNAs of other sobemoviruses, with the exception of the LTSV sat-RNA, which is capable of forming a stable hammerhead (Fig. 4E) and undergoing cleavage in both polarities.

Evolutionary considerations

The RYMV sc-RNA and LTSV sat-RNA are divergent in both host range and geographic distribution: the RYMV sc-RNA has only been isolated from a virus that replicates in monocot species in Africa, while the LTSV sat-RNA is naturally associated with viruses that replicate in dicot species in Australasia and North America. The sequence identity between such divergent RNAs may be a result of a shared RNA predecessor. Alternatively, it is

conceivable that a historical interaction between these RNAs (*e.g.,* from co-infection of a single host) could result in an intermolecular exchange of genetic information; such a model has been suggested for domain-like homologies within several families of viroids (Keese and Symons, 1985). In either case, the maintenance of the 50-nt terminus region with such a high level of conservation may be an indication that it serves an indispensable function in the replication of these RNAs. For instance, this region also includes a GAUUUU hexamer that is conserved, in both sequence and structure, among all the sat-RNAs associated with sobemoviruses. Being the only sequence that is common in these RNAs, it has been suggested to have a possible central function in sat-RNA replication (Davies *et al.,* 1990).

Based on structural similarities in their hammerhead ribozymes, the CarSV and newt RNAs appear to have shared a common RNA ancestry (Daros and Flores, 1995). Consequently, similarity in the hammerhead structure of these RNAs and that of the RYMV sc-RNA, in addition to an apparently similar mode of activity (*i.e.,* using a "double" hammerhead to stabilize an otherwise unstable single hammerhead), may suggest an ancient relationship among these divergent RNAs. Moreover, the presence of apparently homologous retroviroid and sat-RNA structures (*i.e.,* in the hammerhead and left terminus) within a single RNA entity makes the RYMV sc-RNA a possible candidate as an evolutionary bridge between these classes of viroid-like pathogens. Of course, the possibility cannot be overlooked that convergent evolution may account for sequence and structural homologies between these RNAs.

Because the amount of sc-RNA encapsidated by RYMV virions is low compared to other viruses (such as LTSV), this small RNA may have only recently become associated with its helper virus (RYMV). Accordingly, other sobemovirus and plant–host interactions may be capable of supporting its replication and packaging (transport) requirements.

MATERIALS AND METHODS

Isolation of virus and viral RNAs

RYMV was isolated from greenhouse-grown rice (*Oryza sativa* cv. Lamont) 3 weeks postinoculation. Symptomatic leaves were harvested and homogenized in ice-chilled 0.2 M sodium acetate buffer (pH 5.0) containing 0.2% β-mercaptoethanol. The sap was expressed through cheesecloth and clarified by stirring with 2% chloroform for 20 min at 4°C. Following one cycle of differential centrifugation (10,000 and 98,000 *g*), the viral pellet was dissolved in 0.2 M sodium acetate buffer (pH 5.0) containing 0.1% sodium azide. After an additional centrifugation at 10,000 *g* the virus was stored at 4°C.

Viral RNAs were isolated from virions by two phenol: chloroform extractions followed by ethanol precipitation. Total virion RNAs were then fractionated with 2 M LiCl into high- and low-molecular-weight RNAs according to Sehgal *et al.* (1993) and stored in ethanol at −20°C. The fraction enriched with the small RNA was subsequently used for cDNA synthesis.

Cloning of sc-RNA using random primers

First-strand cDNA synthesis was according to AbouHaidar and Paliwal (1988) with some modifications. Two hundred nanograms of fractionated RNA was heat denatured with 0.5 μg random hexamer DNA primers (Pharmacia) and then mixed in a 100-μl reaction volume consisting of 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 5 mM DTT, 1 mM each dNTP, 50 μCi [α-^{32}P]dCTP, 10 μg/ml of actinomycin D, 60 units of RNAguard (Pharmacia), and 400 units of M-MLV reverse transcriptase (BRL). The reaction temperature, initially at 25°C for 10 min to allow the primers to anneal, was increased to 42°C at a rate of 1.5°/min and maintained at 42°C. After 1 h the reaction mixture was centrifuged (1500 *g* for 2 min) through a Sephadex G50 column.

Second-strand synthesis, using half of the desalted first-strand reaction, was performed at 25°C for 2 h in a 100-μl reaction volume containing 40 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, 6 mM DTT, 0.1 mg/ml acetylated BSA, 1 mM each dNTP, 3.6 units RNaseH and 13 units of Sequenase Version 2.0 (Amersham). To generate blunt ends, 10 units of T4 DNA polymerase (NEB) was added and the reaction mixture further incubated at 37°C for 30 min. Unincorporated nucleotides were removed by centrifugation (1500 *g* for 2 min) through a Sephadex G50 column. Double-stranded cDNA was adapted for cloning by dCTP-tailing (AbouHaidar and Paliwal, 1988) and annealed to *Pst*I-linearized, dGTP-tailed pBS(+) (Stratagene).

Recombinant plasmids were recovered by blue/white selection of transformed *Escherichia coli* DH5α grown on X-gal and ampicillin-containing media. Twenty-four clones were screened by Southern hybridization (according to Ivanov and AbouHaidar, 1992) with a randomly primed, [α-^{32}P]dATP-labeled DNA probe (Eweida *et al.,* 1989) for genomic RYMV (full-length clone kindly provided by C. Brugidou). All 24 clones did not hybridize with the genomic probe, and 5 of these were subsequently used to generate a mixed [α-^{32}P]dATP-labeled DNA probe for Northern hybridization of both fractionated and total viral RNAs. Hybridization to the small RNA confirmed the identity of these clones. Three of the 24 putative sat-RNA clones were then subcloned, either as *Xma*I–*Pvu*II fragments (into pBS(+) digested with *Xma*I–*Sma*I) or by digesting cDNA and the pBS(+) polylinker with *Sma*I. Five of these subcloned constructs, when sequenced using Sequenase Version 2.0 (in reactions performed according to the supplier's instructions), produced overlapping partial sequences.

Use of RT-PCR to generate full-length clones

A pair of sense and antisense overlapping primers (P2, 5′-GCGATCCCGGGTCGACCTGGGCGGCTAG-3′, and P3, 5′-GCGATCCCGGGTGAAATTCCTGGGTGCCTC-3′; non-complementary sequences to sc-RYMV are underlined; Fig. 1A), each harboring an *Xma*I site at the 5′ end, were synthesized based on the circular sequence obtained using random primers. The two back-to-back primers were used for "one-tube" RT-PCR (M. Krejdan, personal communication) such that 100 ng of each primer was added to 0.1 μg of total viral RNA in a 100-μl reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.25 mM each dNTP, 11 units of RNAguard, 100 units of M-MLV-RT, and 1 unit of *Taq* DNA polymerase. The reverse transcription reaction was performed at 42°C for 45 min followed by a 1-min denaturing step at 94°C and 30 cycles of PCR (94°C for 30 s; 64°C for 1 min; 72°C for 1 min). Double-stranded DNA products were subsequently subjected to electrophoresis on agarose gels to confirm their size, restriction digested with *Xma*I (NEB), and ligated using T4 DNA Ligase (NEB) to *Xma*I-linearized pBS(+). Sequencing confirmed the identity of a 220-nt clone as a full-length monomer of the RYMV sc-RNA. One clone containing a head-to-tail cDNA dimer was generated by restriction digesting the monomer clone with *Xma*I and religating in a reduced volume. Dimer-sized clones were selected by digestion with enzymes in the pBS(+) polylinker which do not cleave the cloned sequence (*Eco*RI and *Hin*dIII). Screening for orientation of the clones was done with digestion at a unique *Pvu*II site and agarose gel electrophoresis of the resultant fragments.

In vitro transcription and cleavage

Runoff transcription reactions were performed according to Sit and AbouHaidar (1993) using either 0.1 μg *Eco*RI- or *Hin*dIII-linearized plasmid DNA and T7 (NEB) or T3 (Promega) RNA polymerase. The polarity of both monomeric and dimeric cDNA clones was such that linearization with *Hin*dIII and transcription with T7 RNA polymerase generated (+) transcripts, while linearization with *Eco*RI and transcription with T3 RNA polymerase generated (−) transcripts.

Autocatalytic cleavage reactions were performed according to Forster and Symons (1987). Transcription reactions were treated with RQ1 DNase at 37°C for 20 min, then deproteinized with phenol–chloroform and ethanol precipitated. The RNA pellets were resuspended in 1 mM EDTA, heated at 80°C for 1 min, and then snap cooled on ice for 5 min. Cleavage buffer [50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM EDTA] was added and the reaction was incubated at 37°C for 60 min. Cleaved RNA was used directly for electrophoresis in 8% polyacrylamide/7 M urea gels.

Primer extension to identify cleavage site

Full-length (+) transcripts from the dimer clone were eluted from agarose gels and ethanol precipitated. Cleavage was performed as above in 10-μl reaction volumes. The cleaved transcript RNA was subsequently subjected to electrophoresis in polyacrylamide gels in the presence of 7 M urea. The 220- and 164-nt products were eluted from gel slices in 200 μl of deionized water, at 60°C for 1 h, followed by ethanol precipitation along with 2 μg of tRNA.

An 18-mer antisense primer (P1: 5′-AAGCTGCCGGAGCACGAT-3′), hybridizing 26-nt downstream from the location of a putative positive-sense cleavage site predicted from the sequence (Fig. 1A), was used for runoff reverse transcription to the end of cleaved transcript RNAs. Annealing of 100 ng of the primer and synthesis of labeled cDNA in the presence of [α-^{35}S]dATP were done under reaction conditions similar to those used for first-strand cDNA synthesis, with the exception that the reaction was performed at 42°C for 45 min. Following the extension reaction, samples were denatured and subjected to electrophoresis with sequencing reactions of the dimer clone primed at the same position (*i.e.,* using the same primer as the reverse transcription extension reaction). The size of the labeled cDNA could be determined by comparing its migration to that of the sequencing marker, thereby allowing identification of the cleavage site.

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